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Chimerization of Antitumor Antibodies via Homologous Recombination Conversion Vectors

Susan Yarnold and H. Perry Fell[1]

Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121

ABSTRACT

Homologous recombination vectors were designed to convert murine hybridoma cell lines expressing IgG3, IgG1, or IgG2a heavy chains into chimeric human IgG1 producers. These conversion vectors included homology both upstream and downstream of the target sequences and consistently resulted in a higher frequency of successful gene targeting than an insertion vector bearing a single region of homology. A human κ light chain conversion vector was also constructed and used to complete chimerization of the anticarcinoma hybridoma cell line BR96. The resulting cell line expressed antigen-specific chimeric antibody at comparable levels to those found in the murine parental cell line. Southern blots confirm that recombination occurred within the upstream and downstream regions of homology for both vectors, resulting in the loss of murine constant region sequences.

INTRODUCTION

mAbs[2] directed against human tumor-associated antigens are well recognized as an important class of molecules with potential clinical utility (1–4). The ability to localize to human tumor cells *in vivo* offers a variety of possibilities for either therapy or imaging. The appropriate Fc portion of the antibody may also allow for tumor cell lysis via complement activation or antibody-dependent cell-mediated cytotoxicity. Drugs, toxins, or radionuclides can be linked to mAbs for targeting and potential therapeutic effect. Radionuclides are also used for imaging.

To date, mAbs have been most readily obtainable via murine immunization and hybridoma technology. Murine mAbs have been administered to human cancer patients demonstrating successful localization *in vivo* (5); however, immunogenicity can complicate continued administration. It has become well appreciated that the constant regions of a given antibody can be replaced with those from another isotype or species (chimeric mAb) with the antigen-binding properties being maintained. Replacement of murine constant regions with those of human ones reduces immunogenicity in cancer patients (6) and is expected to maximize effector functions when interfacing with the human immune system. Although a complementarity-determining region-replaced or "humanized" (7) form of the antibody may ultimately be preferred for *in vivo* use, in many instances chimeric antibodies may be just as efficacious and certainly serve as readily attainable tools with which to establish therapeutic utility.

Both we and others have shown that the *Ig* locus within a hybridoma genome can be targeted via homologous recombination (8–11). We have previously demonstrated that the heavy chain constant regions of a murine hybridoma that recognizes a human tumor-associated antigen can be switched to those of human IgG1 in a rapid fashion using such an approach (8). The previous strategy made use of a single region of homology to the murine *IgH* locus flanking the human constant regions to target them appropriately. This vector also encoded the *neo* gene for dominant selection. Thus, by simply transfecting the linearized vector directly into the murine hybridoma cell line expressing the desired specificity, selecting for drug resistance, and screening the resulting transfectants for the expression of human heavy chain, clones were quickly established that produced human IgG1 chimeric heavy chains (8).

However, because no genetic material is apparently lost during this process, the insertion of exogenous DNA into the *Ig* locus via homologous recombination results in the effective duplication of the homologous sequences used for targeting at either end of the site of integration. It has been shown that in some cases the duplicated sequences can allow for the reverse reaction to occur, namely the excision of the exogenous DNA (12), resulting in the reconstitution of the wild-type locus.

Here we describe the construction of "conversion" (gene replacement) vectors which bear homologous sequences both 5′ and 3′ to the human constant regions to be introduced into the murine *Ig* locus. We find that these vectors consistently target more efficiently than insertion vectors, resulting in a higher frequency of cell lines expressing chimeric protein. The BR96 cell line expresses a highly tumor-reactive and internalizing mouse monoclonal antibody desirable for clinical trials as a human chimeric drug conjugate (13). Conversion vectors were, therefore, used to switch both the heavy and light chain constant regions to that of human γ_1 and human C_κ, respectively. The resulting cell line was shown to produce antigen-specific chimeric *Ig*. Southern blot analysis demonstrated that integration of both vectors has occurred via conversion events.

MATERIALS AND METHODS

Plasmid DNA Constructs. All DNA manipulations were done by standard procedures (14). The heavy chain conversion vector T/hγ_1/mγ_3 is composed of the following sequences 5′–3′: The 2.3-kilobase *Hind*III fragment that contains J_{H4} and the murine heavy chain enhancer sequences, the 2.8-kilobase *Hind*III-*Pvu*II fragment bearing the genomic DNA sequences that codes for the human γ_1 constant region (15), the *neo* gene for selection in mammalian cells (16), and the coding sequences for the murine γ_3 constant region contained on the 4.7-kilobase *Fsp*I-*Hind*III fragment (17).

The murine γ_{2a} and γ_1 isotype-specific conversion vectors, T/hγ_1/mγ_{2a} and T/hγ_1/mγ_1, were constructed by replacing the murine γ_3 sequences in T/hγ_1/mγ_3 with the 4.7-kilobase *Xho*I-*Eco*RI fragment bearing the murine γ_{2a}- constant region (18) or the *Bam*HI-*Eco*RI fragment that contains murine γ_1 (19), respectively. Each of the fragments bearing murine constant regions begin within the coding region of CH1 and are therefore nonfunctioning genes. The heavy chain vectors were designed such that the vector sequences can be separated from the rest of the plasmid by digestion with the appropriate enzymes.

The light chain conversion vector LCC/D^R contains the following sequences 5′–3′: The 2-kilobase *Pst*I-*Xmn*I fragment bearing the murine κ enhancer (20), the 2.8-kilobase *Eco*RI fragment that contains the genomic sequences that code for human C_κ (21), a mutated pSV2 *DHFR* gene (22) for selection in mammalian cells, and the ~1.7-kilobase *Bam*HI fragment downstream of the sequences that code for murine C_κ (23). The vector was designed such that digestion with *Pst*I separates vector from targeting sequences.

Cell Lines. The murine hybridoma cell line BR96 produces an IgG3 antibody that binds most colon, breast, ovarian, and lung carcinomas (24). Isotype switch variants that produce IgG1 and IgG2a antibodies with identical speci-

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[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: mAb, monoclonal antibody; C_κ, κ constant region; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate.

ficities were isolated from the IgG3 parental cell line (kindly provided by M. MacLean and D. Yelton, Bristol-Myers Squibb, Seattle, WA). 96.5 is a hybridoma cell line that produces a mAb that binds the human melanoma-associated antigen p97 (25). The L6 hybridoma cell line produces mAb which reacts with carcinomas of the breast, lung, ovary, and colon (26). The tumor cell line H3396 was derived from a metastasis of a human breast carcinoma (27). All of the above cell lines were maintained in Iscove's modified Dulbecco medium containing 10% (v/v) fetal bovine serum (GIBCO BRL, Grand Island, NY).

DNA Transfections. All cell lines were transfected by electroporation (using a Gene Pulser; Bio-Rad, Richmond, CA). Approximately 0.8×10^7 cells in 0.8 ml of phosphate-buffered saline were mixed with 25 μg of plasmid DNA and subjected to a 960-μF pulse at 200 V. Forty-eight h after transfection the cells were plated in 96-well plates at 10^4 cells/well. One 96-well plate was set up at 10^3 cells/well to determine transfection frequencies. Cells that were transfected with the heavy chain vectors bearing the *neo* gene were selected in media supplemented with 1 mg/ml of G418 (GIBCO BRL). Selection for the cells that were transfected with the light chain conversion vector containing the mutated *DHFR* gene was carried out in 40 nM methotrexate (Sigma Chemical Co., St. Louis, MO).

Detection of Chimeric Antibodies. Culture supernatants were screened for the presence of human IgG1 by ELISA using a goat anti-human IgG (Antibodies, Inc., Davis, CA) as a capture reagent and goat anti-human IgG conjugated to HRP (American Qualex, LaMirada, CA) to detect. Supernatants from cells transfected with the light chain vector were screened in an ELISA where goat anti-human κ (Tago, Burlingame, CA) and goat anti-human κ conjugated to HRP (Tago) were the capture and detection reagents, respectively. ELISA plates were developed with 3-, 3′-, 5-, 5′-tetramethylbenzidine chromagen (Genetic Systems, Seattle, WA).

Southern Analysis. Genomic DNA was prepared as follows. Approximately 5×10^7 cells were pelleted and resuspended in 10 ml of lysis buffer (0.5% SDS; 100 mM NaCl; 20 mM Tris, pH 7.6; 10 mM EDTA; 0.1 mg/ml of proteinase K; 0.01 mg/ml of RNase). After a 2-h incubation at 37°C, cell lysates were extracted with phenol:chloroform (50:50). DNAs were precipitated with 25 ml of ethanol. Pellets were washed once with 70% ethanol and resuspended in 0.5 ml of 10 mM Tris, pH 7.5–1 mM EDTA.

Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN) and New England Biolabs (Beverly, MA). Ten μg of genomic DNA were digested, electrophoresed through a 0.5% agarose gel, and transferred to a nylon membrane according to the manufacturer's recommendations (Boehringer Mannheim). Hybridizations and chemiluminescent detection was carried out according to the manufacturer's protocol (Boehringer Mannheim). The polymerase chain reaction was used to synthesize digoxigenin-labeled probes (28) with a thermal cycler from Perkin Elmer Cetus (Norwalk, CT). Polymerase chain reaction was done for 33 cycles of 1 min at 94°C, 2 min at 45°C and 3 min at 72°C. The general location of the probes is shown (see Fig. 4). The heavy chain conversion vector, T/γ_1/γ_3, was used as the template for the synthesis of the human γ_1 and murine γ_3 probes. The sense and antisense oligonucleotides used for the human γ_1 probe were 5′-GCGAGACTGTGATGGTTCTTT-3′ and 5′-GTGACGTGGTGTGTGATCCCC- 3′, respectively. The murine γ_3 sense and antisense oligonucleotides, respectively, are 5-CACAGTCTCATCTGTCCTGCAGTC-3′ and 5′-TAACCTTCTTCTTGGACATTTGTT-3′. The light chain conversion vector, LCC/D^R, was the template DNA for the D and E probes. The sense and antisense oligonucleotides, respectively, were D: 5′-TGCGATTTCGCGCCAAACTTGACG-3′ and 5′-CCCATGGTCTTATAAAAATGCATA-3′ and E: 5′-TCTCCCACAACCTTGAGCCTTCTA-3′ and 5′-TCACCTTTGATGTAAACATCATTT-3′. The template DNA used for the synthesis of the human C_κ probe contained the *Eco*RI fragment (described above) that bears the human C_κ sequences. The sense and antisense oligonucleotides were 5′-AGAATGGCTGCAAAGAGCTCCAACA-3′ and 5′-TGACTTCGCAGGCGTAGACTTTGTG-3′, respectively. A plasmid containing the *Bam*HI fragment bearing murine C_κ (20) was the template DNA used in the murine C_κ probe polymerase chain reaction. The sense and antisense oligonucleotides were 5′-CATACCATCCTCTGTGCTTC-3′ and 5′-TGATTACTACCATTTAGT-3′, respectively.

SDS-PAGE and Western Blot Analysis. Purified chimeric BR96 protein was compared to murine IgG3 BR96 and chimeric L6 (29). The proteins were reduced with 2-mercaptoethanol and subjected to SDS-PAGE 4–20% gradient gels (Novex, San Diego, CA) which were either stained with Brilliant Blue G (Sigma) or transferred to nitrocellulose in 25 mM sodium phosphate buffer for 2 h at 150 mA. The nitrocellulose filters were blocked overnight at 4°C in blocking solution (2% nonfat dry milk in phosphate-buffered saline) and then incubated for 3 h at room temperature with goat anti-human IgG-HRP conjugate (American Qualex) diluted 1:3000 in blocking solution and goat anti-human κ-HRP conjugate (Tago) diluted 1:1000 in blocking solution or goat anti-mouse IgG and IgM (γ, μ, and light chains)-HRP conjugate (Tago) diluted 1:1000 in blocking solution. Filters were then washed in 50 mM Tris (pH 7.5)-150 mM NaCl-0.05% Tween 20. The filters were developed with enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).

Competition Binding Analysis. Five $\times 10^5$ H3396 lung carcinoma cells were incubated with IMDM/10% FBS (background) or 5 μg/ml of biotinylated chimeric BR96 diluted in IMDM/10% FBS with or without competitor for 1 h at 4°C in a final volume of 100 μl. The cells were then washed three times with 750 μl IMDM/10% FBS and incubated with 50 μl of a 1:1000 dilution of avidin-FITC (Tago) (in IMDM/10% FBS) for 0.5 h at 4°C. The cells were then washed twice and analyzed by flow cytometry. The linear fluorescence equivalence (LFE) was used to calculate the binding ratio

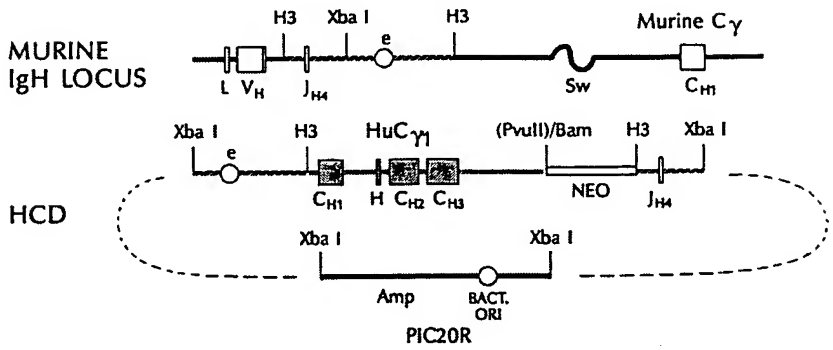
$$\textit{Binding ratio} = \frac{\textit{LFE of sample}}{\textit{LFE of background}}$$

RESULTS

Vector Design. Insertion vectors make use of a single region of homology to drive recombination. Thus, as described previously (8), we chose a segment of DNA that lies downstream of the variable region but upstream of the IgH switch region for the heavy chain insertion vector (Fig. 1*A*). This region thus remains common to any murine genome-producing antibody, regardless of antigen specificity or isotype. Heavy chain conversion vectors, however, were designed to target specifically the human γ_1 constant region to either the murine γ_3, γ_1 or γ_{2a} locus (Fig. 1*B*). The same 2.3-kilobase fragment containing J_{H4} and the murine heavy chain enhancer, reported previously for the insertion vector, was used for 5′ homology flanking the human constant-region exons for each of the isotype-specific conversion vectors. The *neo* gene was placed just 3′ to the human sequences followed by sequences homologous to the particular isotype to be targeted (*i.e.*, γ_3, γ_1, or γ_{2a}).

For the 3′ region of homology, the exon encoding C_H1 was truncated at the 5′ end for each of the murine heavy chain isotypes, resulting in a loss of the splice acceptor site. Thus, recombination within both the 5′ and 3′ regions of homology should result in the loss of endogenous sequences (*i.e.*, the switch region and 5′ end of C_H1) coincident with the introduction of both the human exons and the selectable marker into the locus. Recombination within any portion of the 3′ sequence renders the endogenous constant regions mute with respect to protein expression. The heavy chain conversion vectors were constructed such that digestion with the appropriate restriction enzymes separates the plasmid vector from the sequences to be introduced and their flanking murine sequences for targeting recombination.

Likewise, a light chain conversion vector was designed to replace the murine C_κ exon with that of human C_κ (Fig. 1*C*). The 5′ homology consisted of a 2-kilobase *Pst*I-*Xmn*I fragment from the region bearing the murine light chain enhancer, and the 3′ homology consisted of a ~1.7-kilobase region located downstream of the murine C_κ exon bounded by *Bam*HI sites. A mutant form of the *DHFR* gene (which allows for selection in cells that retain the endogenous *DHFR* gene) (22) was incorporated downstream of the human C_κ exon but preceding the region of 3′ homology to allow for selection of transfected cells. To do sequential transfections, the LCC/D^R vector was designed such that the sequences being targeted to the light chain locus shared no homology with those already targeted to the heavy chain locus. The pUC-derived sequences necessary for DNA propa-



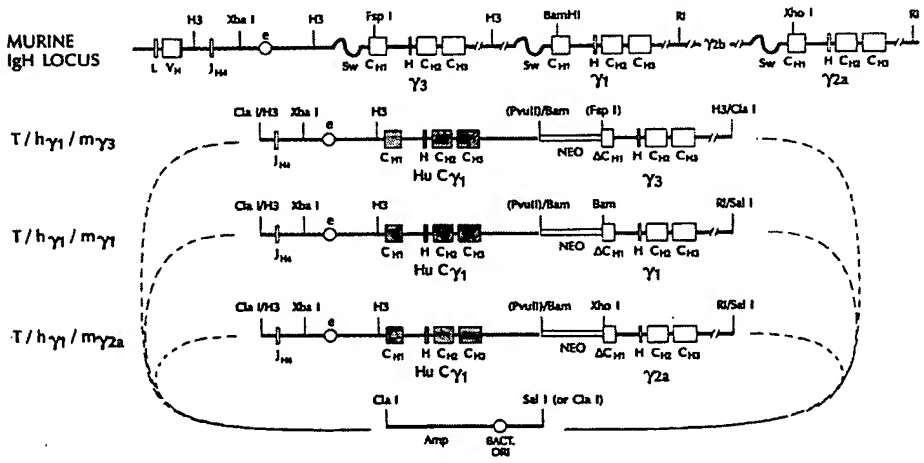
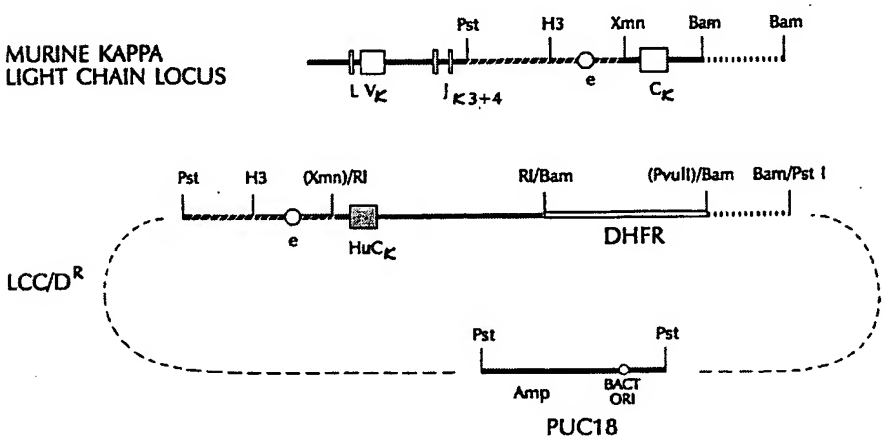


Fig. 1. Homologous recombination vectors. A diagrammatic representation of the five vectors is shown relative to the genomic locus to be targeted. *A*, heavy chain insertion vector; *B*, three heavy chain conversion vectors; *C*, light chain conversion vector. *Empty boxes*, murine exons; *shaded boxes*, human exons. The regions located between V and C exons used for targeting are *hatched*. ----, junctions between recombination sequences and the plasmid vector. Restriction site designations are: *Bam* (*Bam*HI), *H3* (*Hind*III), *RI* (*Eco*RI), *Pst* (*Pst*I), or as indicated. Restriction sites in parentheses were lost during cloning. *L*, leader; *V*, variable region; *J*, joining segment; *e*, enhancer; S_w, switch region; *C*, constant region; *Hu*, human; *amp*, ampicillin; *bact. ori.*, bacterial origin of replication.



gation in bacteria that are present in both heavy and light chain vectors can be dissociated from the targeting sequences by digestion with the appropriate enzymes (see Fig. 1). The *neo* and mutant *DHFR*-selectable markers have different promoters and polyadenylate signals; thus, there are no homologies within the sequences being targeted to the light and heavy chain loci that could lead to recombination between the vectors themselves.

Comparison of Insertion *versus* Conversion Targeting Frequency. The heavy chain conversion vectors were compared with the insertion vector for the relative efficiency of integration via homolo-

Table 1 *Insertion versus conversion targeting frequency*[a]

Cell line	Isotype	Vector	No. of G418- resistant colonies screened	No. of colonies expressing human Ig	Frequency of homologous *versus* nonhomologous integration events
L6	2a	TKneo/γ_1[b] (ins)	4032	2	0.05
L6	2a	T/γ_1/γ_{2a} (conv)	3891	28	0.72
96.5	2a	HCD (ins)	5059	8	0.16
96.5	2a	T/γ_1/γ_{2a} (conv)	2650	20	0.75
BR96	2a	HCD (ins)	1224	2	0.16
BR96	2a	T/γ_1/γ_{2a} (conv)	1075	11	1.02
BR96	3	HCD (ins)	4128	3	0.07
BR96	3	T/γ_1/γ_3 (conv)	3744	13	0.35
BR96	1	HCD (ins)	5376	16	0.30
BR96	1	T/γ_1/γ_1 (conv)	6147	30	0.49

[a] The ability to mediate homologous recombination is compared for heavy chain conversion *versus* insertion vectors. The frequency of homologous *versus* nonhomologous integration events is

$$100 \times \frac{\text{no. of integration events resulting in expression of human IgG}}{\text{total no. of integration events, } i.e., \text{ number G418-resistant colonies}}$$

[b] TK*neo*/γ_1 is an insertion vector which uses the identical region of homologous sequence for targeting as HCD but includes plasmid sequences which integrate into the genome upon recombination. TK*neo*/γ_1 was derived from pCMV/γ_1 (8) by replacing the promoter and polyadenylate signal for the *neo* gene in pCMV/huγ_1 with those used for the *neo* gene in the HCD and conversion vectors.

gous recombination (Table 1). Transfection was performed by electroporation into cell lines expressing tumor-specific antibodies of the appropriate isotype, then selection was performed in media containing G418 to identify cells that had stably integrated the plasmid. Transfection frequencies were determined by plating at lower cell density (*i.e.*, 10^3 cells/well *versus* 10^4 cells/well). Cells surviving drug selection were screened for the production of chimeric antibody, which can occur only by integration of the human constant regions downstream of an appropriate transcriptional promoter, translational initiation site, and splice donor sequence provided by the endogenous functionally rearranged variable-region gene segment. Although results were variable between cell lines for the insertion vector, the conversion vectors consistently resulted in a high frequency of successful targeting events for each of the five cell lines tested, with frequencies observed as high as 1 in 100.

A clonal cell line was established from the BR96 IgG3 parental cell line transfected with the T/hγ_1/mγ_3 conversion vector expressing chimeric heavy chain. This cell line was then transfected with the LCC/D^R conversion vector (Fig. 1*C*) to complete the chimerization for both the heavy and light chain. The transfection was carried out as described in "Materials and Methods." After selection in 40 nM methotrexate, drug-resistant cells were screened for production of human C_κ. Of the 1267 transfection events screened, 11 were positive in a human κ ELISA.

SDS-PAGE and Western Blot Analysis. A single clone was isolated that produced 50–90 μg/ml of chimeric antibody as detected by ELISA of 7- to 10-day spent culture supernatants. This compares favorably with the parental cell line which produces 89–156 μg/ml under similar conditions. Protein A was used to purify antibody (30) from this cell line and the IgG3 parent, as well as the chimeric L6 antibody generated through conventional cloning methodology (29). SDS-PAGE analysis showed that the BR96 chimeric heavy chain was exactly the same size as both the parental IgG3 and the heavy chain from chimeric L6 (Fig. 2*A*). Chimerization of the light chain, however, was found to alter slightly its electrophoretic mobility.

Western blot analysis was also performed, demonstrating appropriate immunoreactivity of both the heavy and light chain (Fig. 2*B* and *C*). The chimeric chains were found to react exclusively with goat anti-human antisera of the correct specificity, and not with goat anti-murine IgG3 or goat anti-murine C_κ antisera.

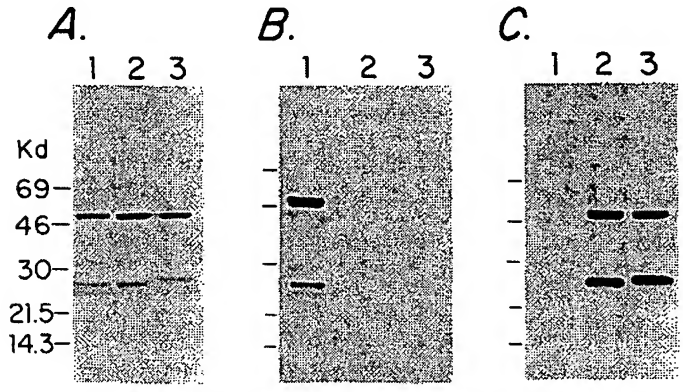


Fig. 2. SDS-PAGE and Western blot analysis. Purified parental murine BR96 IgG3 (*Lane 1*), chimeric L6 IgG1/κ (*Lane 2*), and chimeric BR96 IgG1/κ (*Lane 3*) are compared on Coomassie-stained SDS-PAGE gel (*A*) and in Western blots (*B* and *C*). Western blots were stained with goat anti-mouse immunoglobulin (H and L chain specific) (*B*) or goat anti-human IgG and goat anti-human κ (*C*) as described in "Materials and Methods." *Kd*, molecular weight in thousands.

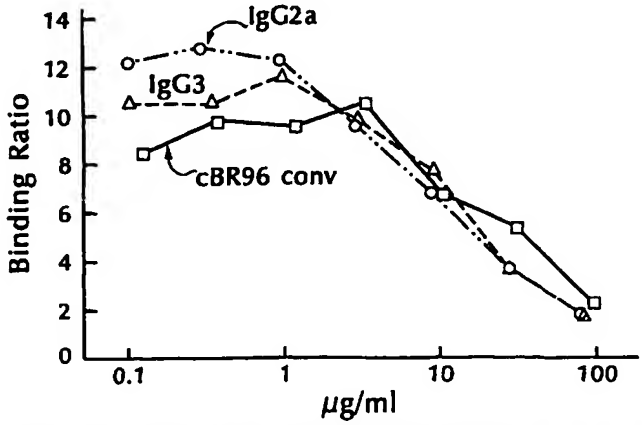


Fig. 3. Antigen binding competition. Binding of purified biotinylated chimeric BR96 to H3396 lung carcinoma cells is shown as a function of increasing concentrations of parental murine IgG3, a murine IgG2a isotype switch variant of BR96, or unmodified chimeric BR96 detected with avidin-FITC on a fluorescence-activated cell sorter as described in "Materials and Methods."

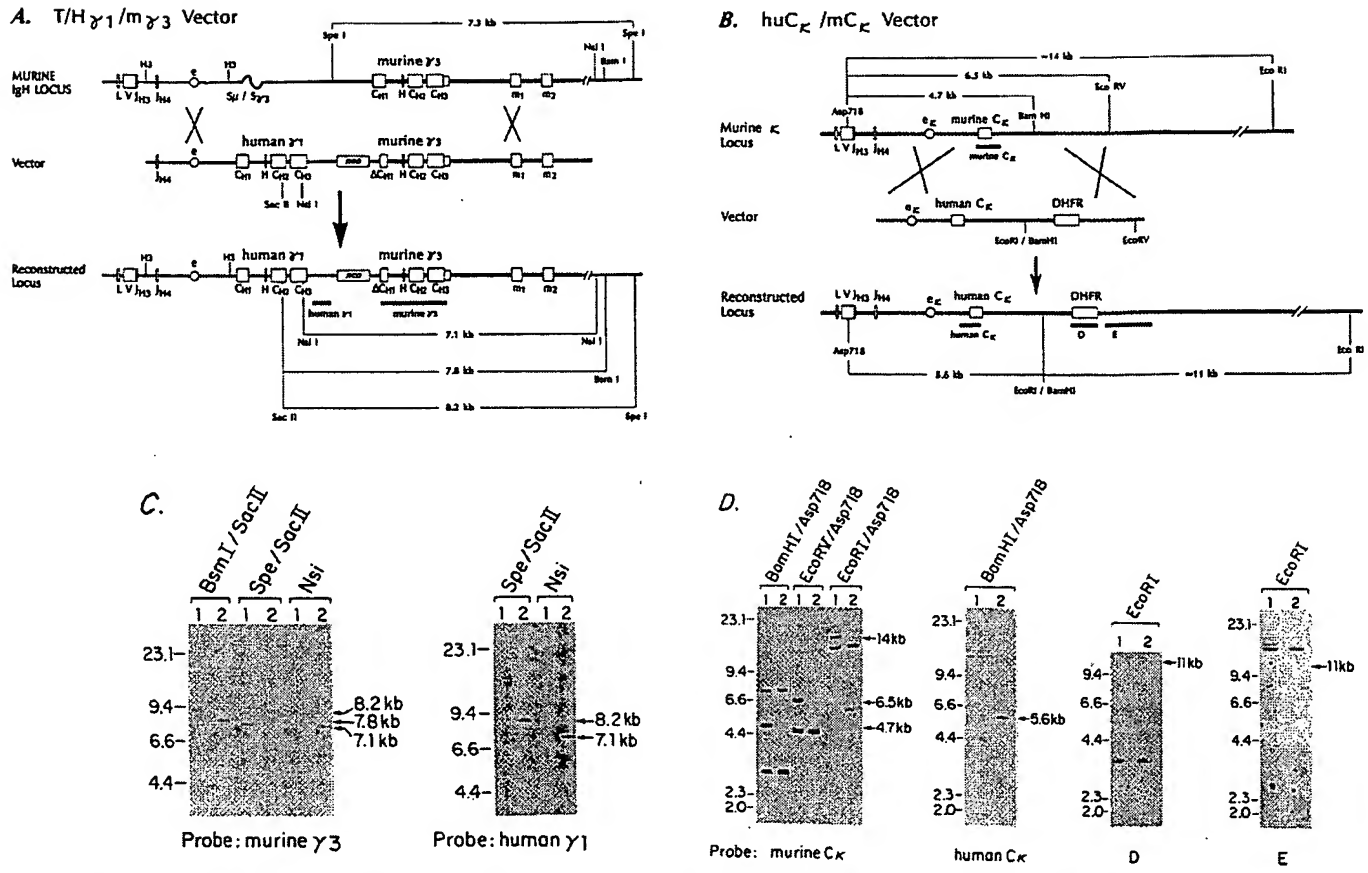


Fig. 4. Southern blot characterization of integration events. Genetic maps of genomic target region, vector, and reconstructed locus for heavy and light chain genes are shown in *A* and *B*, respectively, with the corresponding Southern blot analysis comparing the parental murine γ_3 BR96 hybridoma (*Lane 1*) to the chimeric producing transfectoma (*Lane 2*) shown below for heavy and light chain genes in *C* and *D*, respectively. The position of DNA probes used for hybridization is indicated below the map of the reconstructed locus by *solid bars*. *kb*, kilobase.

Chimeric BR96 Retains Antigen Specificity. Chemical conjugation of the parental murine IgG3 antibody repeatedly resulted in precipitation. Therefore, the chimeric protein was biotinylated and used in competitive binding experiments (Fig. 3). Glutaraldehyde-fixed H3396 cells bearing the BR96 antigen were used as targets to which the biotinylated antibody could bind and then be detected by incubation with avidin-FITC on a fluorescein-activated cell sorter. This binding was found to be inhibitable in a dose-dependent fashion by coincubation with unlabeled parent or chimeric antibody, confirming that the chimeric antibody maintains antigen specificity.

Both Heavy and Light Chain Vectors Integrated by Conversion Events. The reconstructed *Ig* loci within the hybridoma cell line expressing chimeric BR96 were characterized by Southern blot hybridization. Because antigen-specific protein of the correct size is produced by these cells, the human constant regions must have integrated downstream of the functionally rearranged variable regions as intended. However, it is possible that this could occur via the 5′ region of homology, with random integration occurring somewhere within the locus for the 3′ end. Because several restriction sites are known both within the vector and outside of the homologous sequences used to target, we can predict the size of fragments that hybridize to specific probes if the 3′ sequences integrated via homologous recombination (Fig. 4, *A* and *B*).

For the heavy chain, three restriction sites, *Nsi*I, *Bsm*I, and *Spe*I, lie just downstream of the region used for 3′ homology. A *Sac*II site present in the human γ_1 constant region allows for double digestion with *Bsm*I or *Spe*I, which should result in 7.8- and 8.2-kilobase fragments, respectively, when probed with murine γ_3, provided that recombination took place within the γ_3 region. As shown in Fig. 4*C*, this was the case. It was also observed that the γ_3 locus within the parental cell line is distinct and maintains, in particular, the 7.3-kilobase *Spe*I fragment expected for the unmodified locus. There is also a *Nsi*I site within the human γ_1 constant region, which after appropriate recombination within the 3′ sequences should lie 7.1 kilobases away from the *Nsi*I site just downstream of the region used for homology. The results shown in Fig. 4*C* also demonstrate that the murine γ_3 sequences of the parent reside on a *Nsi*I fragment of >10 kilobases, whereas those of the transfectant lie on a fragment of 7.1 kilobases, as predicted.

A Southern blot of the same digests was also hybridized with a probe specific for the 3′ end of human γ_1. This probe is expected to hybridize to the same fragments as that of murine γ_3 in the previous digests. This was found to be true, and the most disparate size fragments were chosen for presentation (*i.e.*, the 7.1-kilobase *Nsi*I and the 8.2-kilobase *Sac*II-*Spe*I fragments Fig. 4*C*). These results demonstrate that the human γ_1 constant region has integrated at a single site, within the γ_3 locus, via recombination within both the 5′ and 3′ region of homology.

There are several loci within the parent and transfected genome which hybridize to a probe specific for murine C_κ (Fig. 4*D*, *first panel*). However, both the heavy and light chain variable regions have been sequenced,[3] revealing that the productively rearranged V_κ segment has a relatively rare *Asp*718 site. Therefore, the productive κ locus should be characterized by a 4.7-, 6.5-, and 14-kilobase C_κ-bearing fragment when digested with Asp 718 and *Bam*HI, *Eco*RV, or *Eco*RI, respectively (see Fig. 4*B*). As shown in Fig. 4*D* these frag-

[3] Unpublished data.

ments were indeed observed within the genome of the parent cell line but were not found in DNA from the transfectant. Therefore, the C_κ exon associated with the productive κ allele was readily identifiable and was found to be absent in the transfected cell line with no new fragments observed in any of these digests. Thus recombination must have occurred on both sides of the C_κ exon present within the expressed transcription unit (*i.e.*, by conversion) resulting in its removal from the locus and elimination from the genome.

Further characterization of the modified light chain locus demonstrated that the human C_κ exon integrated at a single site, placing an internal *Bam*HI site the expected 5.6 kilobases away from the *Asp*718 site within the expressed V_k gene segment (Fig. 4*D*, *second panel*). Recombination within the 3′ region of homology was characterized by digestion with *Eco*RI. Probes specific for either DHFR (probe D) or the region used for 3′ homology (probe E) both hybridize to the predicted 11-kilobase fragment within the transfected genome. Probe E again identifies three κ loci within the genome of the parent cell line. However, in the transfected cell line, one of these fragments has now been shifted to the expected size of 11 kilobases. Thus, the murine C_κ exon has been deleted from the functional κ transcriptional unit and replaced with the human C_κ exon by virtue of a double recombination event.

DISCUSSION

We have shown that homologous recombination offers a rapid and efficient means of converting hybridoma cell lines expressing mAbs with therapeutically relevant specificities into cell lines expressing human chimeric antibody with the same antigen binding characteristics. Previous work demonstrated that for the heavy chain this could be accomplished by placing a single region of homology upstream of the human constant region exons, allowing for insertion into the murine *IgH* locus by homologous recombination (8). Insertion events, however, result in the effective duplication of the region of homology at either end of the site of integration. Such direct duplication of sequences can provide substrates for subsequent recombination events (12), which could lead to the effective removal of the newly introduced sequences. Although such events are expected to be infrequent, in the present example removal of the human constant region exons (as well as the inclusive selectable marker gene) would result in the reconstitution of the murine *Ig* gene locus leading to the potential production of murine antibody again.

It is well appreciated, however, that homologous recombination may also be effected via conversion events, which require regions of homology on either side of the targeted mutation site. Thus, separate vectors must be constructed for targeting to the specific heavy chain isotype being expressed by a given hybridoma. We, therefore, designed vectors bearing homology both 5′ and 3′ of the human constant region exons and selectable marker gene for three of the more prevalently expressed isotypes and demonstrated that each is more efficient than the "universal" insertion vector. In the case of the IgG3 vector and cell line we have also demonstrated that recombination can occur within both the 5′ and 3′ regions of homology, resulting in the effective elimination of the murine exons from the locus, and in fact, from the entire genome, because no new fragments were observed in Southern blots using probes specific for the murine exons. This obviously eliminates the possibility of subsequent recombinations within the locus giving rise to reversion to the expression of murine antibody.

Using insertion vectors, frequencies between cell lines ranged from 0.05 to 0.30%, suggesting that the efficiency of mediating this process can vary significantly among cell lines. The L20 cell line reported previously (8) consistently yielded homologous *versus* nonhomologous frequencies of $\geq$0.3% using a similar vector. However, when conversion vectors are used, the variation between cell lines falls between 0.35 and 1.0%, and for four of the five cell lines tested these frequencies are 5–10 times higher than that observed with the insertion vector. Thus, not only do the conversion vectors result in a consistently higher frequency of homologous events but there also appears to be less variation between cell lines. This could be due to improved frequency associated with the increased amount of substrate DNA coupled with an overall maximum potential of around 1%. Alternatively, these results may reflect a fundamental difference between the process of insertion *versus* conversion homologous recombination. Regardless, the conversion vectors reported here provide a rapid and consistent means for genetically modifying the *Ig* locus within hybridoma genomes expressing antibodies of interest.

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